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EDITORIAL

Molecular biology: the key to personalised treatment in radiation oncology?

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ABSTRACT. We know considerably more about what makes cells and tissues resistant or sensitive to radiation than we did 20 years ago. Novel techniques in molecular biology have made a major contribution to our understanding at the level of signalling pathways. Before the “New Biology” era, radioresponsiveness was defined in terms of physiological parameters designated as the five Rs. These are: repair, repopulation, reassortment, reoxygenation and radiosensitivity. Of these, only the role of hypoxia proved to be a robust predictive and prognostic marker, but radiotherapy regimens were nonetheless modified in terms of dose per fraction, fraction size and overall time, in ways that persist in clinical practice today. The first molecular techniques were applied to radiobiology about two decades ago and soon revealed the existence of genes/proteins that respond to and influence the cellular outcome of irradiation. The subsequent development of screening techniques using microarray technology has since revealed that a very large number of genes fall into this category. We can now obtain an adequately robust molecular signature, predicting for a radioresponsive phenotype using gene expression and proteomic approaches. In parallel with these developments, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) can now detect specific biological molecules such as haemoglobin and glucose, so revealing a 3D map of tumour blood flow and metabolism. The key to personalised radiotherapy will be to extend this capability to the proteins of the molecular signature that determine radiosensitivity.

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Molecular biology developments have, over the past 20 years, provided us with a remarkable array of techniques, enhancing our understanding of how tumour and normal tissues respond to radiation damage. As these techniques grow increasingly sophisticated, their application should, in theory, present opportunities to improve the effectiveness of radiotherapy.

However, as we look at how radiotherapy is performed today we see a discipline founded on 100 years of practice-based, empirical development, recently enhanced by impressive advances in dose delivery and image-guided procedures. These developments have brought us to a point where dose deposition is already highly tailored, to a tolerance of ~2% for most tissues of the body, which is much more accurate than any pharmaceutical agent. Yet, are we really delivering dose where it needs to go for maximal therapeutic gain?

Basic radiobiology and the five Rs

The interaction of high energy X-ray photons with tissue leads to the ejection of fast electrons from

molecules (predominantly water), which in turn go on to generate a wide spectrum of secondary electrons, photons and free radicals. The most reactive of these radicals, OH, is capable of creating further radicals of macromolecules. If these are essential for cellular function, as in the case of DNA, then cell biology will be perturbed and this may lead to cell death.

These processes have been known for at least 50 years, but there is still much to learn about the full spectrum of damaging species resulting from an incident high-energy photon. It is also well established that the initial consequences at the cellular level of free radical formation can be fundamentally modified by two main conditions: the oxygen tension and the concentration of free radical scavengers such as glutathione. How the cell handles and responds to its accumulated damage then depends on more complex processes involving DNA repair and activation of death signalling pathways. Consequences at the tissue level are more complex still and their relevance to radiation oncology was initially explained in terms of the four Rs of Radiobiology: DNA repair (enzymic), reoxygenation (of previously hypoxic cells), repopulation (cell proliferation), redistribution (to phases of the cell cycle with differing radiosensitivity). Later, it was recognised that sensitivity between cell lines could also vary for reasons that could not be explained by the four Rs, so a fifth R, intrinsic radiosensitivity, was

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added [1]. These five concepts could explain in general terms the variability in cellular and even tissue response, but in most cases really tell us little about the mechanisms regulating the response to radiation exposure at the molecular level. The more recent application of molecular techniques to this subject has added extra complexity, and this has been comprehensively reviewed [2].

Predictive testing based on traditional radiobiology

Each of the five Rs is capable of contributing a substantial dose modifying factor (2–5, though most are not strictly dose modifying) to the eventual outcome of radiotherapy. It is therefore logical that an assessment of these parameters in individual patients could be of enormous predictive value.

However, this has not proved to be true, with attempts to develop predictive assays based on measurement of these five parameters being met with mixed success. But we should remember that the science underlying the five Rs was aimed at providing a framework to aid understanding of new phenomena in radiation biology rather than predicting outcomes. Furthermore, the lack of success may be because there are only small quantitative differences between many normal tissues and human tumours, and a large degree of overlap in their heterogeneity.

Hypoxia

It has been known for many years that hypoxia, measured using a variety of direct and indirect methods, correlates strongly with the outcome of all cancer therapies. This is not just limited to patients receiving therapies that are known to be oxygen-dependent in their cytotoxic action, such as radiotherapy [3].

There is now overwhelming evidence the hypoxia regulates cancer outcome by several mechanisms including increasing inflammation, promoting malignant progression and directly reducing the effectiveness of therapies [4]. The application of molecular techniques has greatly enhanced our understanding of how these phenomena are mediated, particularly the role of the master regulator HIF-1.

For prostate cancer patients treated with radiotherapy or surgery only, increased staining for HIF-1 alpha expression and the key downstream mediator, vascular endothelial growth factor (VEGF), were significant predictors of a shorter time to biochemical failure [5]. Furthermore, a 99-gene hypoxia gene signature derived from head and neck tumours might turn out to be radiobiologically relevant in terms of stratifying patients for radiation treatment [6], as would the meta-signature highlighted by the same group [7].

Cell kinetics

Cell cycle parameters vary widely between individual human tumours and it is logical to imagine that the duration of the cell cycle or of its distinct phases should

have some influence on the outcome of radiotherapy. After all, we know that cells are more sensitive to radiation in some phases than others and that proliferation is related to the rate of tumour regrowth during and after treatment. Few studies have been large enough individually to give a statistically significant answer. However, one multivariate analysis of head and neck cancer patients from 11 different European centres showed clearly that no cell kinetic parameter could be relied upon to predict local control [8].

Intrinsic radiosensitivity

It might seem self-evident that the intrinsic radiosensitivity of tumour or even normal cells derived from cancer patients should correlate with the outcome of treatment. Not only has this been hard to demonstrate, but different endpoints for DNA damage do not correlate well with each other [9].

The most extensive studies have involved measurement of the surviving fraction of tumour cells from cervix cancer patients in vitro after 2 Gy (SF2) [10–12]. A clear correlation was found, though it required careful selection of the appropriate cut-off value for SF2 (0.42) to discriminate clearly between good and poor outcomes. A similar result was also obtained, though not consistently in head and neck cancer [13–15].

However, the limitations of these approaches are probably both technical and biological. Primary cultures from human tumours are very hard to grow and even when they do form colonies, the plating efficiencies are around 1%. This would not matter if tumours contained uniform cells populations, but the discovery of cancer stem cells supports the view that the clinical response of tumours is dominated by a very small, resistant subpopulation of cells that can proliferate indefinitely [16]. The remainder of the tumour cells are largely irrelevant. Thus, SF2 values are averages across a very large number of cells and so are not so representative of the resistant clones that determine cure or relapse. As well as these difficulties, the SF2 assay has another crucial weakness – even when a tumour sample does generate colonies, the result takes up to a month to obtain.

The general acceptance of the importance of the double strand break as a key DNA lesion determining cell fate after irradiation has led to an assessment of its repair as a surrogate marker of radiation sensitivity (see Hennequin et al 2009 [16] for review). Results have been variable: in one study of ten human tumour cell lines, DNA end-binding complexes (indicative of initiation of repair) correlated with SF2 in primary fibroblast cultures and human tumour cell lines [17]. A more definitive assay may be the scoring of chromosome aberrations and there is clear evidence from work on human cell lines that they may predict for cell survival [18, 19].

Functional genomics and molecular responses to radiation exposure

We now know that exposure to ionising radiation, in common with other DNA-damaging agents, initiates a complex series of up and down regulations of genes

interacting through many pathways. The pioneering work in this area was carried out more than 20 years ago by Fornace and colleagues, who showed, using cDNA library screening, that key genes, GADD45A and p21^(CIP1/WAF1), were up-regulated by ionising radiation [20, 21]. The pivotal role of the p53 regulator became apparent around this time and data suggested that multiple pathways downstream of this master regulator must be important in the response of cells to ionising radiation. Key proteins include GADD45, CDKN1A and MDM2. Initial studies showed that they were induced by a large dose (20 Gy) of X-rays, but not in all cell lines and not in a predictable, p53-dependent manner [20].

The advent of microarray technologies provided novel tools for the identification of changes in gene expression in response to ionising radiation. The first application of this technology to radiation response again used large (20 Gy) doses and allowed the relationships between a variety of genes, many unknown and many regulated by p53, to be mapped out [21]. The same group also showed that microarray analysis could also detect differential gene expression in blood cells in response to radiation doses in the clinically relevant therapeutic range of 2 Gy or less. Many of these genes, which we know are involved in apoptosis or cell cycle checkpoints, show a dependency on dose rate [21].

Microarray analysis has also been instrumental in revealing the mechanisms involved in mediating bystander killing in unirradiated cell populations adjacent to and sharing a common medium with irradiated ones. This includes evidence for the involvement of connexin 43, a protein with a role in gap junction communication, and cyclooxygenase [22]. Bystander responses can also be transmitted via the medium that has supported the growth of irradiated cells [23]. While no comprehensive profiling has been carried out p53, p21 MDM2, CDC2, Cyclin B1 and RAD51 are all significantly modulated in bystander cells.

Considerable effort has been invested in evaluating the expression of individual genes and gene products as markers of biological response to radiotherapy. This raised expectations that biomarkers would more accurately predict outcome than traditional approaches that relied on size, dissemination, stage and grade. While biomarkers for response to chemotherapy are increasingly recognised *e.g.* HER2/neu in breast cancer (see Lawrence et al, 2008 [24] for review) their application to radiotherapy planning is much less advanced. The role of the cell cycle and DNA repair regulator EGFR in determining radiosensitivity has been documented in tumours in several sites (colorectal, brain and head and neck), but the correlation is not wholly consistent. Similar associations have been reported for members of the p53 gene family and genes regulated by p53 [25, 26]. Also, several genes including, cyclin D1, TS, TP, DPD, and Her-2/neu have been shown, using quantitative RT-PCR techniques, to be predictors of response, survival, and recurrence in patients treated with radiochemotherapy for squamous cell carcinoma of the oesophagus [26].

As the number of genes implicated in response to radiotherapy increases, several groups have used microarray analysis to obtain a global signature indicative of radioresponsiveness/resistance in colorectal cancer [27–29]. Several other gene signatures have emerged in

relation to the radioresponse of cervical, breast and head and neck tumours (reviewed in West et al 2007 [30]) and more recently in breast cancer [31, 32]). However, overlap between these gene signatures is minimal, the statistical significance has been questioned and there are no large-scale, randomised trials yet published to fully validate the usefulness of any of these signatures in the different tumour types. What is needed is a gene expression model that predicts intrinsic radiosensitivity and treatment response in a broad spectrum of cancer patients. A useful approach may be to determine differential expression of key genes with known roles in processes that could impact on biological response to radiation (see Begg 2009 [33] for recent review). An important step in that direction was taken in a recently published study [33]. Radiosensitivity was modelled as a function of gene expression, tissue of origin, ras (mut/wt), and p53 status (mut/wt) in 48 human rectal, head and neck and oesophageal cancer cell lines. This group identified 10 key or “hub” genes involved in pathways central to the regulation of cell signalling.

We are accustomed to seeing well-defined relationships (*e.g.* linear quadratic) between radiation dose and endpoints for cell damage such as cell survival, but it became clear early on that changes in the expression of radiation-modulated genes do not generally exhibit these relatively simple dependencies. For example, a transcriptional response that is exclusive to low doses has been reported in several studies [35–38].

Gene expression studies such as these have helped to identify pathways of interest, but we need to be aware that cellular responses are mediated at the protein level such that translational regulation, post translational modification and degradation of proteins must add additional levels of complexity to the genomic responses identified by microarrays.

As well as using gene expression as a radiobiological endpoint, other investigators have used genotyping to link germ-line single nucleotide polymorphisms (SNPs) within both normal and tumour tissue, with a view to assessing normal tissue radiation toxicity and tumour response [30, 39]. With particular reference to tumour response, the studies demonstrated that genetic variations associated with DNA repair and apoptosis appear to be important. Four large studies are now under way to fully validate markers for normal tissue radiation toxicity [40, 43], though large-scale validation of SNPs that might be useful predictive markers of tumour radio-responsiveness is still lacking. However, studies like the normal tissue radiation toxicity (RAPPER) study, might allow an increase in tumour dose for radiation-tolerant patients, increasing their probability of local recurrence-free survival. Furthermore, if a relationship exists between tumour and normal tissue radiosensitivity, this will further enhance the potential of genetic profiling in the management of radiotherapy patients.

Application to archived samples

Many hospitals have extensive archives of formalin fixed tumour and normal tissue material for which the patient outcome is known. Theoretically, this resource should be amenable to genomic, RNA and proteomic

analysis, providing the opportunity for the identification of key pathways involved in the radiation response of tumour and normal tissue to clinically relevant irradiation schedules. To date, the number of studies using this material is rather limited, mainly because of the difficulties of analysing samples containing very small amounts of heavily degraded DNA.

However, methods have been developed for profiling of SNPs in DNA from paraffin-embedded tissue [44, 45]. The problem of sample size has been addressed by using whole gene amplification techniques and can be used to detect changes in gene copy number [45]. Bead arrays have also been used as a novel, high throughput method for determining DNA methylation and gene silencing.

Changes in gene expression at the RNA level can also be determined in paraffin-embedded sections using real time PCR combined with proteinase digestion, and further enhanced by laser-assisted microdissection to focus on regions of interest and maximise the amount of message in the sample [47].

Protein analysis can also yield information from archival material [48]. Immunohistochemical staining of tissue sections with specific antibodies can be successful if combined with pre-treatment using antibody retrieval methods, such as heating. This approach can also be used to extract proteins from fixed material in a manner that is suitable for 2D gel electrophoresis.

Alternatively, removal of paraffin from sections and enzyme digestion have improved the effectiveness of matrix-assisted laser desorption/ionisation liquid chromatography/mass spectrometry analysis. Protein interactions are fundamental to the activation of pathways in response to cytotoxic insults including radiation. These interactions can be identified using a system that exploits proximity ligation of oligonucleotides that are attached to specific antibodies [49, 50].

This kind of information obtained using gene expression and proteomic approaches [51] has made it increasingly possible to identify regulation of specific pathways with radiotherapy outcome. This offers the real possibility of using that knowledge to inform patient care in the form of personalised therapy at the molecular level. The contribution that proteomics, including mass spectrometry could make in translating cell biology into clinical practice is discussed in a recent review [52].

Biomarkers and functional imaging

In many respects, the holy grail of personalised treatment planning is to use non-invasive functional imaging technology to achieve "biologically conformal treatment" [24]. This will require the integration of reliable biomarkers with functional imaging using PET or MRI. While we are a long way from being able to detect levels of expression of genes or their products using remote imaging methods *in vivo*, diffusion-weighted MRI (DCE-MRI) has been used successfully as a predictor of the response of brain, and colorectal tumours, while [^{18}F]FDG-PET data was of prognostic value in lung, gastric, oesophageal, liver, breast, head and neck, and cervical cancer (see Harry et al [53] for recent review).

However, currently, the evidence that either biomarkers or functional imaging are superior to anatomical imaging using CT in patient management is not compelling, at least for head and neck cancer [54].

Molecular techniques are facilitating a rapid expansion in our understanding of the signalling pathways that regulate the radiation response of normal and malignant cells. In parallel, advances in imaging technology, particularly in MRI and PET, are increasing the range of functional markers that can be quantified in tissues in real time.

However, there is still some way to go before non-invasive assessment of an individual patient's tumour, based on molecular markers for specific pathways, will be possible. A few years ago, the mathematical complexity of combining a large number of parameters in each cell with different levels of expression according to each unique position within a tumour and also the problem of temporal changes during protracted treatments would have been beyond the computational power available. Enormous computing power can now be brought to bear on the problem and, combined with advanced computational techniques, offers some expectation of success.

However, complex algorithms are only as good as the inputs entered into them and we still face difficulties in assigning weights to the multiple parameters that would be needed, even if they can be measured accurately. For example, epigenetic effects due to concomitant vascular or immunological pathologies may override the predictions of purely genetic characterisations.

Clearly, it will be many years before a fully integrated approach to personalised treatment can be introduced to clinical practice. In the meantime, though, elements of this concept could contribute to treatment planning in the near future.

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